Short Communication

Microsatellite loci in the tetraploid spined loach, *Cobitis biwae*, and cross-species amplification in four related species

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ABSTRACT. Fifteen microsatellite loci were identified in the tetraploid spined loach, *Cobitis biwae* (Teleostei: Cobitidae). Among these, 14 were polymorphic (5-31 alleles) and showed moderate to high cross-species amplification transferability in four related species, *Cobitis matsubarai*, *Cobitis taenia*, *Misgurnus anguillicaudatus*, and *Misgurnus fossilis*. The loci, described herein, will be useful for population genetics, phylogeny, parentage analysis, and detection of hybridization among *Cobitis* species.

Key words: Hybrid; Polyploid; SSRS; Enriched library

INTRODUCTION

Spined loaches of the genus *Cobitis* (Cobitidae, Cypriniformes) are fresh water fishes, widely distributed in Europe and Asia. They are species complex that result from the natural process of hybridization, unisexual reproduction, and polyploidization (Saitoh et al., 2010; Janko et al., 2012; Choleva et al., 2014). The genus *Cobitis* has attracted interest owing to its unusually complicated and poorly recognized genome constitution, which is a consequence of hybridization and genome duplication events (Bohlen and Rab, 2001).

The isolation of nuclear markers, such as microsatellite markers is helpful for tracing hybridization events in *Cobitis* complexes, and has been previously conducted in some European *Cobitis* species (De Gelas et al., 2008). However, to date, no microsatellite marker has been described for *Cobitis* distributed around Asia.

One of the Japanese *Cobitis* complexes is *C. biwae*, which comprises the diploid and tetraploid forms having 48 and 96 chromosomes, respectively (Kobayashi, 1976; Ueno and Ojima, 1976). The diploid form is distributed throughout the northern and central Honshu and on the Pacific slope of Shikoku Islands, whereas the tetraploid form occurs in the western Honshu and Shikoku and its distribution is limited to the Seto Inland Sea slope (Kimizuka and Kobayashi, 1983; Kitagawa et al., 2003). *C. biwae* could be an appropriate model, not only for zoogeographic studies (Bohlen and Rab, 2001), but also for evolutionary process or recent tetraploidization due to natural gene duplication (Kusunoki et al., 1994; Kitagawa et al., 2003). In the present study, we report the first set of microsatellite loci for *C. biwae* and the levels of their polymorphism among tetraploid individuals. Additionally, all the loci were tested for cross-species amplification in four other related Cobitidae species.

MATERIAL AND METHODS

Fifty-eight tetraploid *C. biwae* individuals captured from the Kamo River, Takehara City, Hiroshima Prefecture, Japan were used in this study. Ploidy status of all the fishes was determined by measuring the DNA content of somatic cells collected from caudal-fin using flow cytometry (Fujimoto et al., 2007). Tetraploidy was determined by the DNA content measured with a Ploidy Analyzer (Partec GmbH, Münster, Germany) using DNA content in the diploid wild type *Misgurnus anguillicaudatus* as the reference (2.53 pg/nucleus; Zhang and Arai, 1996). The DNA content of *C. biwae* (5.75 ± 0.15 pg/nucleus) was in the range of other tetraploid *Cobitis* (Vasil'ev et al., 1999; Juchno et al., 2010). Genomic DNA was isolated from the dorsal fin clip following the standard phenol-chloroform protocol. Microsatellite enriched library was constructed using the DNA isolated from one individual by magnetic beads hybridization selection protocol (Glenn and Schable, 2005). Briefly, approximately 5 mg DNA was digested with *Hae*III (Roche, Basel, Switzerland). After enzymatic digestion, the digested DNA was size selected (500-1.500 bp) using QIAquick gel extraction kit (Qiagen, Hilden, Germany). The recovered DNA was dephosphorylated and ligated to SNX linker (Hamilton et al., 1999) and then amplified by PCR using SNX forward primer. A hybridization step was performed for the enrichment library construction. Purified linker ligated DNA was hybridized to biotinylated $(CAT)_8$ and $(GAT)_8$ probes (hybridization temperatures were about 5 degrees below the Tm of each probe). Subsequently, the hybridized DNA was captured using streptavidin-coated magnetic beads (Dynabeads® Magnetic Beads; Life Technologies, Carlsbad, CA, USA) and subjected to stringent washes to remove the unhybridized DNA. The

recovered DNA was amplified by PCR using the SNX forward primer (initial denaturation for 2 min at 92°C, followed by 25 cycles of denaturation for 45 s at 94°C, annealing for 1 min at 62°C, extension for 1 min at 72°C, and a final extension for 30 min at 72°C). Amplified fragments were ligated into pCR4-Topo vector and transformed into TOP10 One Shot chemical competent *Escherichia coli* cells (Life Technologies). A total of 96 colonies were screened by PCR using M13(-20)-F and M13-R primers, and sequenced using the BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) with M13(-20) forward primer on an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems). Primers, containing sufficient flanking regions, were designed for 35 microsatellite containing sequences using the Primer Premier 6.0 software. All putative microsatellite loci were PCR screened in four *C. biwae* individuals. Finally, 21 primer pairs were selected based on agarose gel visualization of the PCR products for further screening by fragment analysis sequence using M13-tailed primers. PCR was performed as described by Morishima et al. (2008). Briefly, 100 ng template DNA was amplified in a final volume of 10 μL reaction mix containing 200 μM dNTPs, 0.3 pmol M13-tailed forward primer, 3.0 pmol reverse primer, 3.0 pmol fluorescence-labeled M13 primer, and 0.5 U *Taq* polymerase (TaKaRa Bio Inc., Kusatsu, Shiga, Japan); thermocycling conditions were as follows: initial denaturation for 3 min at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 56°C, extension for 30 s at 72°C, and a final extension for 45 min at 72°C. PCR products were sequenced using formamide with GeneScan LIZ-500 size standard (Applied Biosystems). The GeneMapper 3.7 software (Applied Biosystems) was used for allele scoring.

ATETRA 1.2 (Van Puyvelde et al., 2010), which was developed to analyze tetraploid microsatellite data, was used to estimate the number of alleles (N_A), expected heterozygosity (H_E), and Shannon-Wiener diversity indices (H').

RESULTS AND DISCUSSION

After fragment analysis, 15 microsatellite loci resulted in unambiguous peak patterns. These loci were used for genotyping 58 tetraploid individuals of *C. biwae*. Although only tri-nucleotide biotinylated probes were used for constructing the libraries, a high rate of di-nucleotide CA/GT repeat motif was found (73%). This finding is consistent with that of a previous study, where di-nucleotide microsatellite motifs were the most abundant form of microsatellites in vertebrates and arthropods (Tóth et al., 2000). Overall, 14 polymorphic primer sets were used, and revealed up to four alleles per locus per individual, confirming tetraploidy. The N_A per locus ranged from 4 (*Cobi26*) to 31 (*Cobi31*), with a mean of 11.4 (Table 1).

The H_E ranged from 0.388 to 0.947 (mean H_E = 0.693), and the H' ranged from 0.811 to 3.160 (mean H' = 1.672). The loci exhibited moderate to high levels of polymorphism (4 to 31 alleles). The transferability of the microsatellite loci, reported herein, was evaluated in four related species: *Cobitis matsubarai* (Kawatana River, Shimonoseki City, Yamaguchi Prefecture, Japan), *Cobitis taenia* (Lake Leginski, Warmia-Masuria Province, Poland), *M. anguillicaudatus* (Kunebetsu River, Nanae Town, Hokkaido Prefecture, Japan), and *Misgurnus fossilis* (Wieprz River, Lubelskie Province, Poland) (Table 2). Moderate (*M. anguillicaudatus* and *M. fossilis*) to high (*C. matsubarai* and *C. taenia*) levels of cross-species amplification were observed. The new microsatellite loci described in this study will be useful for population genetics, phylogeny, parentage analysis, and hybrid detection among *Cobitis* species.

Table 1. Genetic characterization of 15 microsatellite loci developed for *Cobitis biwae*.

Locus	Primers (5'→3')	Dye	Repeat motif	Size (bp)	N_A	N	H_E	H'	Accession No.
Cobi5	F: CATCAGACCAACCTGTAAGG R: TCATCCCTACACTCATCC	VIC	$(A)_{11}$	267-300	12	48	0.842	2.079	KC573043
Cobi6	F: GGGTTCAATTTCCAGAGAA R: TTTGAACAGCGTGCAGTG	NED	$(CA)_{14}$	204-499	13	58	0.489	1.242	KC573044
Cobi13	F: GGTGTGACTTCATTTCG R: TACAGTACTTGTACCTCAAAGG	NED	$(GT)_{15}$	206-308	15	54	0.770	1.906	KC573045
Cobi17	F: ATCAGTGTCATAGGCACA R: GGACTCAATCATGGTTACAC	VIC	$(CA)_{12}$	198-271	22	50	0.907	2.651	KC573046
Cobi19	F: GAGGTGACAGGTGAGTGA R: CTAATTTGCACCCATTTGGT	PET	$(CA)_6$	232-261	6	56	0.415	0.875	KC573047
Cobi21	F: TTTCAAGTCCAAGAACAGTC R: AGGGTGATAACTGAGAATGA	VIC	$(GT)_5$	217-287	6	54	0.388	0.811	KC573048
Cobi22	F: ATCCAGCAGGTTCTGTCT R: AAGATTCAGATGGCAAGAGA	VIC	$(CT)_{11}$	178-188	6	57	0.703	1.419	KC573049
Cobi24	F: AGAACGCGAAATTGGCAGT R: TCTCTCTACCTCATTCGCT	VIC	$(GT)_{15}$	247-283	8	48	0.812	1.804	KC573050
Cobi26	F: AATCATCCAACAGGTGTCTGC R: GGTGGATGTGAATGTGACT	VIC	$(GAT)_6\ldots(A)_{12}$	180-294	4	58	0.645	1.162	KC573055
Cobi27	F: GCACAGCCATGATACCTTA R: AACGGATGCTTGACAGAC	VIC	$(GT)_{13}$	252-415	14	51	0.819	2.004	KC573051
Cobi30	F: GACAGAGACCTTTATTTCTG R: AGATGTCACGATGCATTTGT	VIC	$(AC)_5(AG)_9$	106-328	7	45	0.749	1.498	KC573052
Cobi31	F: CCAGAGTCTTGCCAGGTA R: CAGGAAGATTGGACGCTAT	NED	$(GT)_{10}$	250-372	31	58	0.947	3.160	KC573053
Cobi32	F: GAGATGATCACTAGAATACATGAGG R: AGAGAAGATCTAGAACTGAG	PET	$(T)_{12}$	120-179	5	58	0.423	0.882	KC573057
Cobi35	F: ATACTGGGACTATGAAGCC R: GACATCACAACGGCATTC	VIC	$(CAT)_9$	256-390	11	56	0.800	1.909	KC573054
Cobi28	F: CCAACTACTCCATATCTTCTTC R: GTGGCGGAGGAACAGTA	VIC	$(CT)_6(GT)_3$	300-328	2	50	-	-	KC573056

F: forward, R: reverse primer sequences, bp: allele size range, N_A: number of alleles, N: number of genotyped individuals, H_E: mean expected heterozygosity, H': Shannon-Wiener diversity indices.

Table 2. Cross-species amplification results of *Cobitis biwae* microsatellite markers in four related species: *Cobitis matsubarai*, *Cobitis taenia*, *Misgurnus anguillicaudatus*, and *Misgurnus fossilis*.

Locus	*C. matsubarai*			*C. taenia*			*M. anguillicaudatus*			*M. fossilis*		
	N	N_A	Size range (bp)	N	N_A	Size range (bp)	N	N_A	Size range (bp)	N	N_A	Size range (bp)
Cobi5	25	6	270-280	7	2	272-273	8	2	265-273	5	1	281
Cobi6	25	4	442-444	7	8	157-250	7	1	105	-	-	X
Cobi13	-	-	O	5	1	239	7	1	252	-	-	X
Cobi17	24	9	225-457	7	1	199	-	-	X	-	-	X
Cobi19	26	6	233-267	7	1	252	8	2	265-273	-	-	X
Cobi21	25	6	232-241	7	2	216-234	7	2	119-326	3	3	132-240
Cobi22	27	5	173-255	8	2	172-186	8	1	174	5	3	314-474
Cobi24	-	-	O	-	-	X	-	-	X	2	1	307
Cobi26	22	3	190-199	6	2	181-195	8	2	116-193	5	3	194-239
Cobi27	-	-	O	-	-	O	-	-	X	-	-	X
Cobi30	-	-	X	7	4	145-190	-	-	X	5	3	154-161
Cobi31	26	4	240-248	8	2	242-244	-	-	X	3	3	188-248
Cobi32	-	-	X	6	1	170	-	-	X	-	-	X
Cobi35	10	4	176-352	-	-	X	8	1	173	-	-	X
Cobi28	4	2	305-307	5	3	313-319	-	-	X	3	4	256-482

N: number of individuals, N_A: number of alleles, bp: allele size range, X: no amplification, O: multiple bands pattern or product of unexpected size.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Bohlen J and Rab P (2001). Species and hybrid richness in spined loaches of the genus *Cobitis* L. (Teleostei: Cobitidae) with a checklist of European forms and suggestions for conservation. *J. Fish Biol.* 59a: 75-89. http://dx.doi.org/10.1111/j.1095-8649.2001.tb01380.x

Choleva L, Musilova Z, Kohoutova-Sediva A, Paces J, et al. (2014). Distinguishing between incomplete lineage sorting and genomic introgressions: complete fixation of allospecific mitochondrial DNA in a sexually reproducing fish (Cobitis; Teleostei), despite clonal reproduction of hybrids. *PLoS One* 9: e80641. http://dx.doi.org/10.1371/journal.pone.0080641

De Gelas K, Janko K, Volckaert FAM, DE Charleroy D, et al. (2008). Development of nine polymorphic microsatellite loci in the spined loach, *Cobitis taenia*, and cross-species amplification in the related species *C. elongatoides, C. taurica* and *C. tanaitica. Mol. Ecol. Resour.* 8: 1001-1003. http://dx.doi.org/10.1111/j.1755-0998.2008.02135.x

Fujimoto T, Sakao S, Yamaha E and Arai K (2007). Evaluation of different doses of UV irradiation to loach eggs for genetic inactivation of the maternal genome. *J. Exp. Zool. A Ecol. Genet. Physiol.* 307: 449-462.

Glenn TC and Schable NA (2005). Isolating microsatellite DNA loci. *Methods Enzymol.* 395: 202-222. http://dx.doi.org/10.1016/S0076-6879(05)95013-1

Hamilton MB, Pincus EL, Di Fiore A and Fleischer RC (1999). Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques* 27: 500-502, 504-507.

Janko K, Kotusz J, De Gelas K, Slechtová V, et al. (2012). Dynamic formation of asexual diploid and polyploid lineages: multilocus analysis of Cobitis reveals the mechanisms maintaining the diversity of clones. *PLoS One* 7: e45384. http://dx.doi.org/10.1371/journal.pone.0045384

Juchno D, Lackowska B, Boron A and Kilarski W (2010). DNA content of hepatocyte and erythrocyte nuclei of the spined loach (*Cobitis taenia* L.) and its polyploid forms. *Fish Physiol. Biochem.* 36: 523-529. http://dx.doi.org/10.1007/s10695-009-9322-x

Kimizuka Y and Kobayashi H (1983). Geographic distribution of karyological races of *Cobitis biwae* (Cobitididae). *Jpn. J. Ichthyol.* 30: 308-312.

Kitagawa T, Watanabe M, Kitagawa E, Yoshioka M, et al. (2003). Phylogeography and the maternal origin of the tetraploid form of the Japanese spined loach, *Cobitis biwae*, revealed by mitochondrial DNA analysis. *Ichthyol. Res.* 50: 318-325. http://dx.doi.org/10.1007/s10228-003-0174-6

Kobayashi H (1976). Comparative study of karyo-types in the small and large races of spinous loaches (*Cobitis biwae*) (in Japanese with English abstract). *Zool. Mag.* 85: 84-87.

Kusunoki T, Arai K and Suzuki R (1994). Production of viable gynogens without chromosome duplication in spinous loach *Cobitis biwae. Aquaculture* 119: 11-23. http://dx.doi.org/10.1016/0044-8486(94)90440-5

Morishima K, Nakayama I and Arai K (2008). Genetic linkage map of the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae). *Genetica* 132: 227-241. http://dx.doi.org/10.1007/s10709-007-9165-2

Saitoh K, Chen WJ and Mayden RL (2010). Extensive hybridization and tetrapolyploidy in spined loach fish. *Mol. Phylogenet. Evol.* 56: 1001-1010. http://dx.doi.org/10.1016/j.ympev.2010.04.021

Tóth G, Gáspári Z and Jurka J (2000). Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Res.* 10: 967-981. http://dx.doi.org/10.1101/gr.10.7.967

Ueno K and Ojima Y (1976). Diploid-tetraploid complexes in the genus *Cobitis* (Cobitidae, Cyprinidae). *P. Jpn. Acad. B* 52: 446-449.

Van Puyvelde K, VAN Geert A and Triest L (2010). atetra, a new software program to analyse tetraploid microsatellite data: comparison with tetra and tetrasat. *Mol. Ecol. Resour.* 10: 331-334. http://dx.doi.org/10.1111/j.1755-0998.2009.02748.x

Vasil'ev VP, Vinogradov AE, Rozanov YM and Vasil'eva ED (1999). DNA content in cells of different forms from unisexual-bisexual complex of spined loaches from genus *Cobitis* and Luther's spined loach *C. lutheri* (Cobitidae). *J. Ichthyol.* 39: 377-383.

Zhang Q and Arai K (1996). Flow cytometry for DNA contents of somatic cells and spermatozoa in the progeny of natural tetraploid loach. *Fish. Sci.* 62: 870-877.